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Linear Mitochondrial DNAs of Yeasts: Closed-Loop Structure of the Termini and Possible Linear-Circular Conversion Mechanisms

NATHALIE DINOUËL,[1] RACHID DRISSI,[1] ISAMU MIYAKAWA,[2] FRÉDÉRIC SOR,[1] SOLANGE ROUSSET,[1] AND HIROSHI FUKUHARA[1]*

Section de Biologie, Bâtiment 110, Institut Curie, Centre Universitaire Paris XI, Orsay 91405, France,[1] and Faculty of Science, Biological Institute, Yamaguchi University, Yamaguchi 753, Japan[2]

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The terminal structure of the linear mitochondrial DNA (mtDNA) from three yeast species has been examined. By enzymatic digestion, alkali denaturation, and sequencing of cloned termini, it was shown that in *Pichia pijperi* and *P. jadinii*, both termini of the linear mtDNA were made of a single-stranded loop covalently joining the two strands, as in the case of vaccinia virus DNA. The left and right loop sequences were in either of two orientations, suggesting the existence of a flip-flop inversion mechanism. Contiguous to the terminal loops, inverted terminal repeats were present. The mtDNA from *Williopsis mrakii* seems to have an analogous structure, although terminal loops could not be directly demonstrated. Electron microscopy revealed the presence, among linear molecules, of a small number of circular DNAs, mostly of monomer length. Linear and circular models of replication are considered, and possible conversion mechanisms between linear and circular forms are discussed. A flip-flop inversion mechanism between the inverted repeat sequences within a circular intermediate may be involved in the generation of the linear form of mtDNA.

In the accompanying report (8), we show that linear forms of mitochondrial DNA (mtDNA) are frequently found in yeast species. These genomes have a defined terminal structure, as judged from their constant gene order with respect to the termini and the presence of homologous sequences at both termini. In this study, we analyzed in more detail the structure of the termini, as this information is essential to know the origin and mode of replication of the linear genomes. In a number of other organelle genomes, linear forms of DNA have also been identified. In some cases, the reality of the linearity might be questioned. In the case of *Pichia pijperi*, which had the smallest linear mtDNA, we could demonstrate, by sequencing, that the termini of the linear molecule possessed a continuous hairpin structure linking the termini of the two strands. The results obtained with two other species also suggested that an analogous structure may form the ends of their linear mtDNAs.

MATERIALS AND METHODS

Strains, media, and most of the analytical procedures are described in the accompanying report (8). Exonuclease III (ExoIII), nuclease S1, and nuclease BAL 31 were obtained from New England Biolabs (Beverly, Mass.) and used according to the supplier's instruction. The pTZ18/19R vector system (Pharmacia France, Saint Quentin-en-Yvelines, France) was used for cloning and enzymatic DNA sequencing. Electrophoresis of denatured DNA was carried out as described elsewhere (21). For electron microscopy, restriction fragments from termini were treated with the gene 32 protein of *Escherichia coli* bacteriophage T4 as described previously (7) and then deposited on a carbon-coated grid that had been activated by glow discharge in the presence of pentylamine as described previously (7). mtDNA denaturation was performed according to the formamide-glyoxal method, and denatured DNA was spread as in the case of native DNA (8).

* Corresponding author.

Nucleotide sequence accession numbers. The sequence data reported have been assigned EMBL accession numbers X67311, X67312, and X67618.

RESULTS

Restriction fragments from the termini are often seen in two forms. When the mtDNAs from *P. pijperi* and *P. jadinii* were digested with restriction enzymes and electrophoresed, each of the terminal fragments, as defined in the restriction maps, appeared as two bands, one main band and another, called the shadow band, which was less abundant and migrated slightly more slowly than the main band (see Fig. 4 of the accompanying report [8]). This observation suggested that there are two different forms of termini. The total mtDNA (*P. jadinii*) was first treated with calf intestinal alkaline phosphatase and labeled with [γ-^{32}P]ATP and T4 nucleotide kinase and was then digested with various restriction enzymes. Autoradiography of Southern blots showed that the labeling occurred selectively at the termini and that the shadow bands were efficiently labeled, whereas the main bands were poorly labeled (Fig. 1). This is because, as shown later, the shadow bands have an open structure with a readily accessible 5′ end, while the main bands have a closed hairpin end (whose weak labeling is thought to be due to possible internal nicks).

Termini are sensitive to BAL 31 and S1 nucleases. The mtDNA of *P. pijperi* was treated with low concentrations of BAL 31 (which has, besides a double-strand exonuclease activity, a strong single-strand endonuclease activity). The DNA was then digested with restriction enzymes. Gel electrophoresis of the digest showed that the terminal fragments were gradually shortened by increasing BAL 31 treatment. At higher enzyme doses, digestion of the terminal fragments continued while the internal fragments always remained unchanged, as expected (Fig. 2). A similar observation was made with *P. jadinii* mtDNA, although in this case the shadow fragments appeared more resistant to BAL 31 or S1 compared with the main terminal fragments (data not

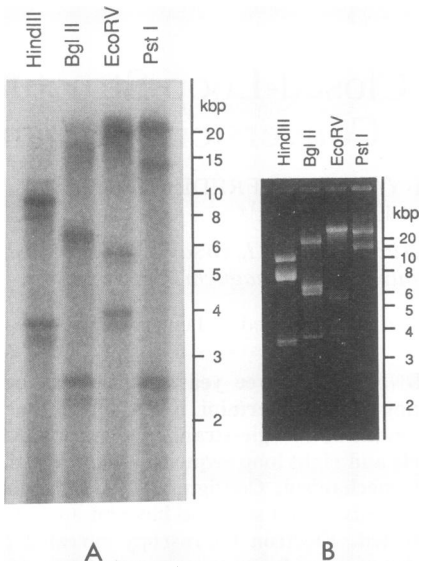

FIG. 1. 5′ labeling of *P. jadinii* mtDNA. Two micrograms of *P. jadinii* mtDNA was dephosphorylated with calf intestinal monophosphatase (2 U) and repurified by phenol extraction. The DNA was incubated with T4 polynucleotide kinase (20 U) in the presence of [γ-^{32}P]ATP for 30 min. After repurification, the DNA was digested with *Hin*dIII, *Bgl*II, *Eco*RV, or *Pst*I and electrophoresed. DNA was then blotted on a nitrocellulose sheet and autoradiographed (A). Note that each terminal fragment shows two components, a strongly labeled band and, below it, a weakly labeled band. The first corresponds to the shadow band, and the second corresponds to the main terminal band. The strongly labeled shadow bands are only weakly visible on ethidium bromide-stained gels (B) because they represent a minor form of the termini. For restriction site maps, see the accompanying report (8).

shown). These experiments showed that the extreme ends, over a few hundreds nucleotides, of the main terminal bands had a structure particularly sensitive to these enzymes, suggesting that the ends had an exposed single-stranded structure.

Alkali denaturation of the terminal fragments indicates the presence of a hairpin loop. The main and shadow bands from *P. pijperi* mtDNA were excised from the gel, denatured with alkali, and electrophoresed in an alkaline agarose gel. Three components were found in the main band: one that had a size twice as long as that of the nondenatured sample, and two other faint bands of unequal size about one-half the size of the first band. The denatured shadow band did not give the double-length component but gave only two bands of unequal size similar to those found in the denatured main band (Fig. 3). When the alkali-denatured fragment was electrophoresed in a neutral gel, the double-length fragment migrated as a single-length molecule. This finding showed that a large fraction of the main terminal band contained a continuous hairpin structure that can snap back at neutrality. The presence of two monomer-size components of unequal length suggested that some of the hairpin structure might have a nick at a nearby specific point, cutting the continuous chain into two unequal segments. Hybridization experiments using the terminal loop sequence (see below) as a probe confirmed that the dimer-length component and the two monomer-size fragments indeed contained the loop sequence (Fig. 3B).

The main terminal bands are resistant to ExoIII. Since the continuous hairpin structure of the termini should be resistant to exonucleases, the mtDNA of *P. pijperi* was treated with ExoIII for various lengths of time (3′-end digestion) and

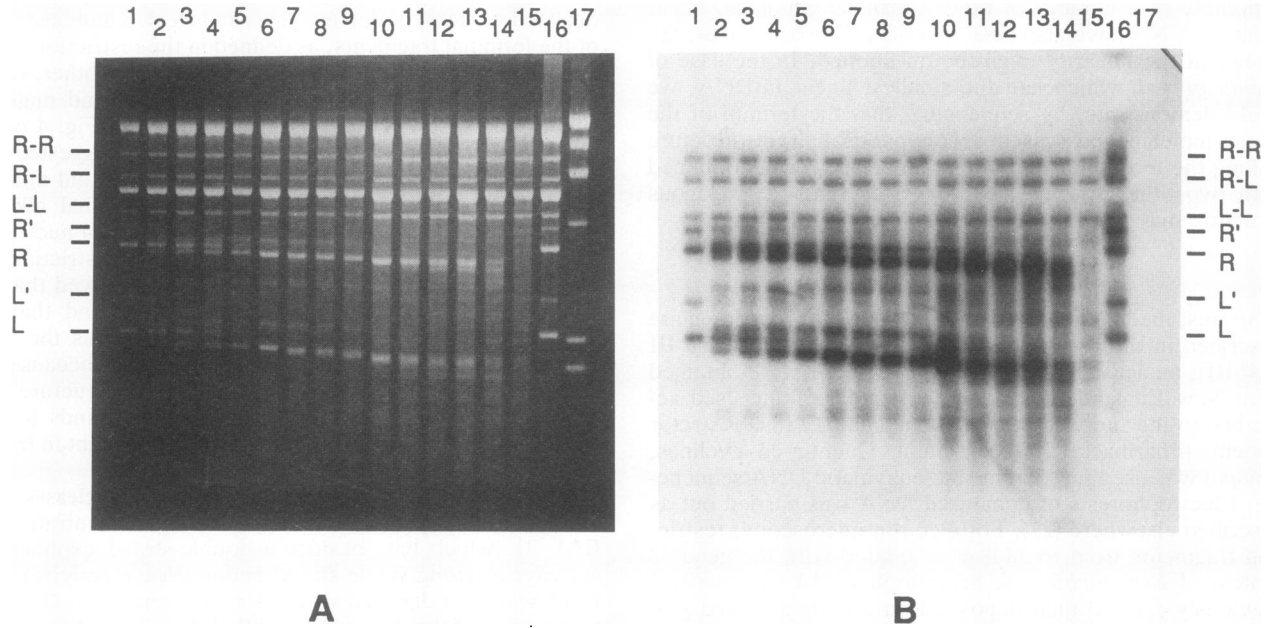

FIG. 2. BAL 31 sensitivity of the termini. mtDNA of *P. pijperi* was treated with increasing concentrations of BAL 31 nuclease before digestion with *Pst*I. The digests were then electrophoresed on an agarose gel. (A) Ethidium bromide-stained gel; (B) autoradiography of the Southern blot after hybridization with a radioactive right terminal fragment as a probe (an internal portion of the inverted terminal repeat sequence). Lanes 1 to 15, mtDNA digested with increasing amounts of BAL 31 (units) as follows: 1, none; 2, 0.001; 3, 0.00125; 4, 0.00167; 5, 0.0025; 6, 0.005; 7, 0.01; 8, 0.0133; 9, 0.02; 10, 0.04; 11, 0.05; 12, 0.067; 13, 0.1; 14, 0.133; 15, 0.2. Lane 16, no BAL 31 as in lane 1: lane 17, molecular weight marker (lambda DNA *Hin*dIII digest). Note that the internal fragments were insensitive to the nuclease, as were the minor bands R-R (right-right) R-L (right-left), and L-L (left-left), which are terminal junction fragments (see text). The right (R) and left (L) terminal fragments, as well as their respective shadow fragments (R′ and L′), were progressively shortened by BAL 31 treatment.

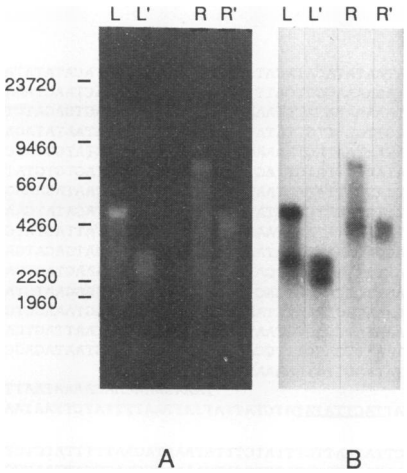

FIG. 3. Alkaline gel electrophoresis of the terminal fragments. mtDNA of *P. pijperi* was digested with *Pst*I and fractionated by electrophoresis. The main terminal bands (L [left] and R [right]) and their respective shadow bands (L′ and R′) were excised from the gel, denatured with alkali, and electrophoresed in an alkaline agarose gel. (A) Ethidium bromide-stained gel; (B) autoradiography of the Southern blot after hybridization with a radioactive single-stranded fragment internal to the right terminal loop sequence as a probe. The initial lengths (kilobase pairs) of the nondenatured fragments were as follows: L, 2.35; L′, 2.85; R, 3.65; and R′, 4.1. In the alkaline gel, L gave a band about twice the size of L′, and the latter resolved into two fragments of unequal size. R and R′ behaved in a similar way. The labeled probe is a 17-base single-stranded oligomer from the right terminus (nucleotides 1889 to 1915 of Fig. 5a). Reference molecular weights (in bases) are indicated at the left.

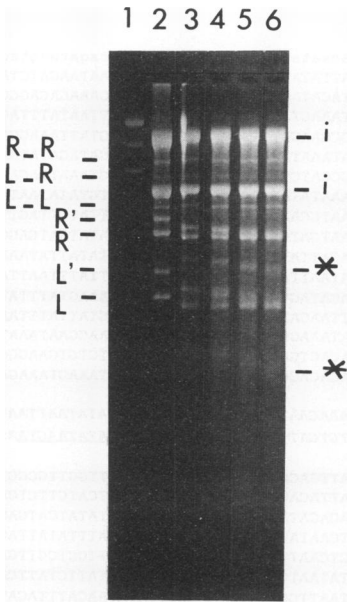

FIG. 4. ExoIII treatment of the termini. *P. pijperi* mtDNA was digested with ExoIII for increasing lengths of time. Each sample was then treated with S1 nuclease for 30 min and digested with *Pst*I. Lanes: 1, molecular weight marker (lambda DNA *Hin*dIII digest); 2, *Pst*I-digested control without ExoIII-S1 treatment; 3, S1 digest without ExoIII; 4 to 6, sample treated with ExoIII for 4, 8, and 12 min and then with S1. Internal fragments (indicated by i) as well as the faint junction fragments (R-R [right-right], R-L [right-left], and L-L [left-left]) were not affected by either ExoIII or S1. The main terminal fragments R and L were sensitive to S1 (lane 3) but not to ExoIII (lanes 4 to 6). Their size decreased slightly due to S1 but remained unchanged in the presence of ExoIII. In contrast, the shadow bands R′ and L′ were progressively shortened by ExoIII. Upon treatment with S1, two diffuse bands (marked by *) appeared which were sensitive to ExoIII. They probably represent the two fragments generated by the attack of S1 nuclease at the supposed single-strand nick within the left and right terminal fragments.

then with S1 nuclease (to eliminate the 5′ overhang generated by ExoIII). The DNA was then digested with a restriction enzyme and electrophoresed. As shown in Fig. 4, ExoIII was indeed unable to digest the main terminal bands, while it progressively shortened the shadow bands. Therefore, the main terminal bands, at least a large fraction of them, have no 3′ ends accessible to ExoIII. The supposedly nicked DNA of the shadow bands should have, in its extended form, a 3′ recessed end since the nick cuts the loop asymmetrically, as shown above.

Cloning and sequencing of the termini. The shadow bands of *P. pijperi* (the right *Xba*I shadow fragment [ca. 3.9 kbp] and the left *Cla*I shadow fragment [ca. 2.0 kbp]) are thought to contain a nicked hairpin structure (which can be schematized as in Fig. 10A, the second form from the top). They were treated with the Klenow large fragment DNA polymerase in the presence of four deoxynucleoside triphosphates (for fill-in synthesis) and cloned into the sequencing vector pTZ19R opened at the cloning sites *Xba*I-*Sma*I and *Sma*I-*Sma*I, respectively. The sequences of the cloned fragments are shown in Fig. 5a. The cloned right and left fragments contained an identical 208-bp unique sequence bordered with inverted sequences on both sides, as expected for a hairpin structure. These complementary sequences formed the inverted terminal repeats of the linear genome. Assuming that no nucleotides were lost at cloning, the position of the original nick used for the fill-in synthesis may be located within the paired part of the DNA, about 500 nucleotides from the base of the terminal loop. However, this estimate should be taken with caution because it does not precisely correspond to the nick position calculated from the unequal lengths of fragments generated in the alkali denaturation experiment shown in Fig. 3. The discrepancy might be due to the inaccuracy of the electrophoretic size estimation of single-strand fragments. Also, the nicks may represent a small gap of unprecise size, as suggested by the fact that the cloned right and left fragments differed by one nucleotide at the junction with the cloning vector sequence.

In the case of *P. jadinii*, the terminal loop has not been sequenced, but the results of S1 digestion of terminal fragments and alkali denaturation of the shadow fragments suggested that the loop sequence would have a length of roughly 400 nucleotides.

The loop sequence can be of either orientation. Complementary single-stranded fragments internal to the right loop sequence of *P. pijperi* mtDNA were chemically synthesized and radiolabeled. Each of these probes hybridized with both right and left terminal fragments of mtDNA with similar intensities (Fig. 6). This finding indicated that the population of mtDNA molecules has the terminal loop in either of the two orientations, with approximately equal frequency.

Terminal inverted repeats. The right and left sequences contiguous to the terminal loops were sequenced. They were identical but in opposite orientation. The length of the inverted repeats in *P. pijperi* was 1,842 bp (Fig. 5a). In *Williopsis mrakii* mtDNA, the inverted terminal repeats were 1,917 bp long (Fig. 5b) and contained several long

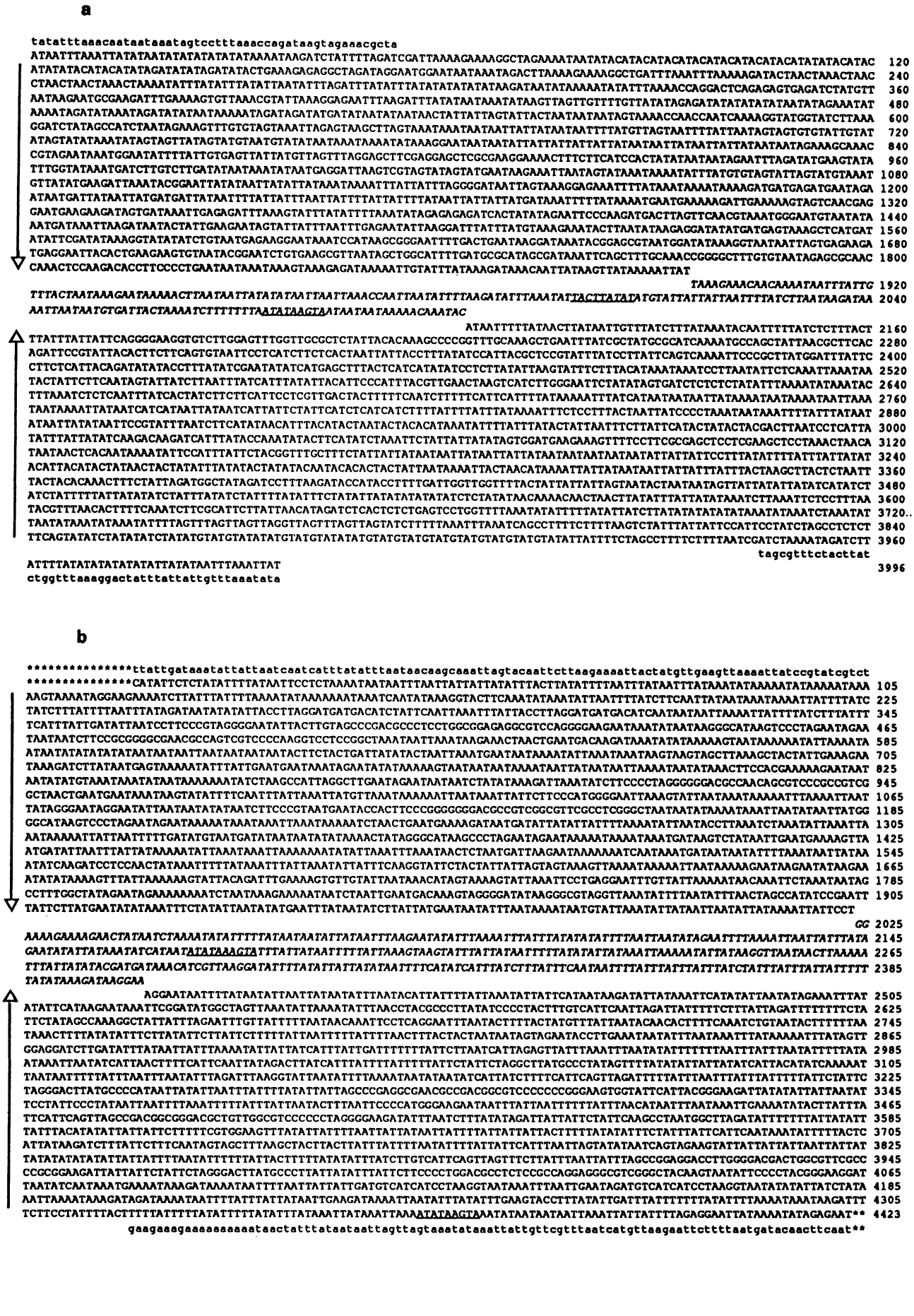
a
b

FIG. 5. (a) Nucleotide sequence of the left terminal hairpin of *P. pijperi* mtDNA. The two vertical arrows indicate the complementary strands of the terminal inverted repeat sequence (positions 53 to 1894 and 2103 to 3944). The block of sequence in italics between the arrowheads is the single-stranded loop sequence forming the end of the molecule (1895 to 2102). Within the loop, the two underlined nonanucleotides, ATATAAGTA and complementary TACTTATAT, are possible transcription initiation sites. The first and the last parts of the sequence are flanked with lowercase letters showing the diverging part after the right terminal repeat (EMBL accession numbers X67311 and X67312 for right and left termini, respectively). (B) Nucleotide sequence of the right terminal hairpin of *W. mrakii* mtDNA (EMBL accession number X67618). The overall presentation is similar to that shown in panel a. The two vertical arrows indicate the complementary strands of the terminal inverted repeat sequence (106 to 2023 and 2403 to 4320). Between the arrowheads is the single-stranded terminal loop in italics (2024 to 2402). The loop sequence was deduced from the sequence determined on the cloned right-right junction fragment (see text). Within the loop, the underlined decanucleotides ATATAAAGTA may be a transcription initiation site. A canonical nonanucleotide ATATAAGTA, also underlined, is present at position 4362, which is thought to be the transcription initiation site of the small-subunit rRNA gene. The flanking lowercase letters show the diverging part following the left terminal repeat of the genome. We noted that the complementary strand of the inverted repeat has one nucleotide mismatch due to a C in excess at position 1969.

internally repetitive motifs. In *W. mrakii*, 50 bp inward from the right inverted repeat begins the small-subunit rRNA gene, and 259 bp inward from the left inverted repeat one finds the end of an open reading frame (ORF1; 492 codons) whose properties will be described elsewhere.

Electron microscopy. Under electron microscopy, the single-stranded termini described above appear as a structure distinct from double-stranded breakpoints. The *P. pijperi* terminal fragment *Hin*dIII (1.3 kbp) was mixed with bacteriophage T4 gene 32 protein and examined under an electron microscope for the presence of single-stranded structure. Forty-six percent of DNA pieces of correct length showed one characteristic end with attached gene 32 protein, while the other end of the same fragment (cut by restriction enzyme) did not show this aspect (Fig. 7). Internal restriction fragments taken as a control showed no proteins attached to their ends (data not shown). This result is consistent with the model of single-stranded termini. On the other hand, a linear mtDNA molecule with a closed loop at both ends should give rise to a single-stranded circle of dimer length upon complete denaturation. To verify this, denatured mtDNA of *P. pijperi* was examined as described above. Among linear fragments, 29 single-stranded circles were observed, 19 of which had a size of 44.2 ± 1.0 kb, suggesting that a large fraction of the *P. pijperi* mtDNA is a single continuous polynucleotide chain. Figure 8 shows one of the single-stranded dimer circles.

Three types of terminal junctions can be detected in small amounts. When restriction enzyme-digested mtDNA was electrophoresed and probed with labeled terminal fragments, three additional bands of low intensity were detected by

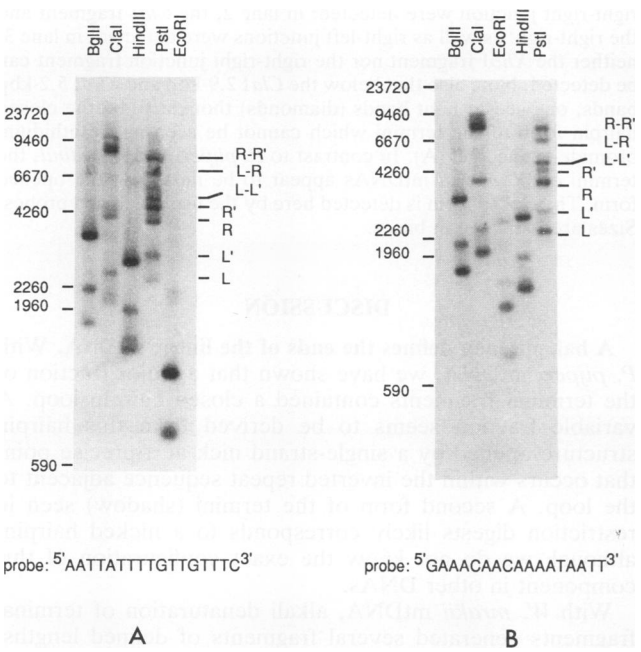

FIG. 6. Orientation of the terminal loop. *P. pijperi* mtDNA was digested with *Bgl*II, *Cla*I, *Hin*dIII, *Eco*RI, or *Pst*I, electrophoresed in duplicate, blotted onto nitrocellulose sheets, and hybridized with terminal loop sequence probes. In panel A, the probe was the same as that specified in the legend to Fig. 3B; in panel B, its complementary sequence was used as the probe. These probes detect specifically the terminal fragments and their derivative forms. These components are indicated in *Pst*I digests by L (left terminal band), L′ (its shadow band), R (right terminal band), R′ (its shadow), L-L′ (left-left junction), R-R′ (right-right junction), and L-R (left-right junction). Note that the two autoradiograms gave essentially the same results, indicating that the single-stranded terminal loops can be in either orientation. Sizes are indicated in bases.

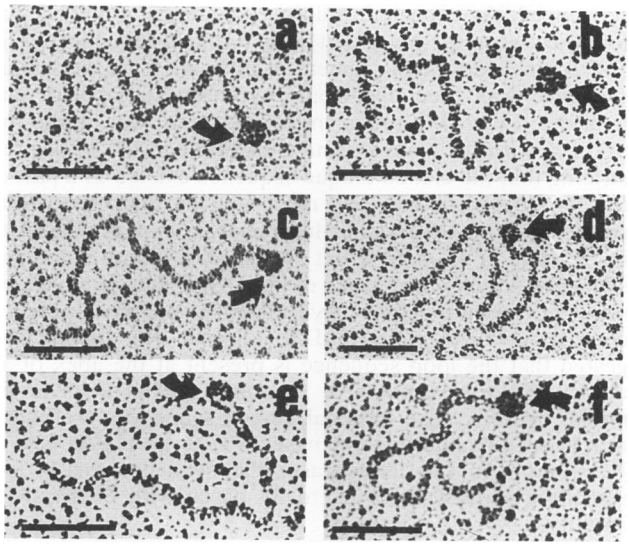

FIG. 7. Electron microscopy of the termini. *P. pijperi* mtDNA was digested with *Hin*dIII and fractionated by agarose gel electrophoresis. The terminal fragments of 1.3 kbp (common to both right and left termini; see restriction map in the accompanying report [8]) were excised from the gel and repurified. The DNA was treated with T4 gene 32 protein as described by Sogo et al. (32) and spread for electron microscopy. Among the fragments of expected size, 46% showed an attached protein particle only at one end (indicated by arrows). Six examples are shown. Bars represent 250 bp.

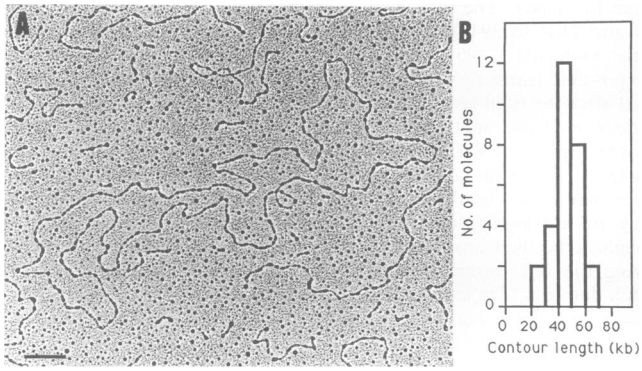

FIG. 8. Single-stranded dimer-length circle. An mtDNA preparation of *P. pijperi* was denatured before spreading. Most of the circular molecules had a contour length of approximately dimer size. (A) Example of the dimer-length circle, 44,000 nucleotides long. The bar represent 1,000 nucleotides. (B) Contour-length histogram of the circles.

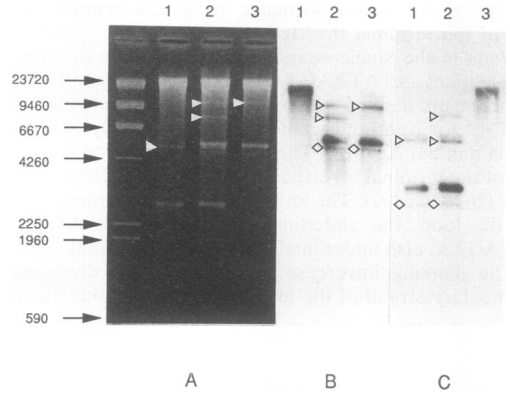

FIG. 9. Detection of left- and right-specific terminal sequences in the junction fragments. (A) *W. mrakii* mtDNA was digested with *Cla*I (lane 1), *Cla*I plus *Xho*I (lane 2), and *Xho*I (lane 3) and stained with ethidium bromide. The junction fragments (weakly stained) are indicated by triangles. (B) The DNA was blotted onto a sheet of nitrocellulose and hybridized with a probe specific for the left arm of the mtDNA (internal *Eco*RI 1.8-kbp fragment within the terminal *Xho*I fragment). In lane 1, neither the right terminal *Cla*I 2.9-kbp fragment nor its junction fragment (twice the size of the terminal *Cla*I fragment) can be detected. In lane 2, the left terminal *Xho*I 5.2-kbp, left-right (*Cla*I [2.9 kbp] plus *Xho*I [5.2 kbp]), and left-left (*Xho*I plus *Xho*I) junction fragments were detected (triangles); in lane 3, the *Xho*I fragment and the left-left junction fragment were detected (triangles). (C) The same blot was hybridized with the right-arm-specific probe (terminal *Cla*I fragment minus inverted terminal repeat). In lane 1, the terminal *Cla*I fragment and the right-right junction were detected; in lane 2, the *Cla*I fragment and the right-right as well as right-left junctions were detected; in lane 3, neither the *Xho*I fragment nor the right-right junction fragment can be detected. Note also that below the *Cla*I 2.9-kbp and *Xho*I 5.2-kbp bands, one sees a faint bands (diamonds) thought to be the closed hairpin form of the termini which cannot be seen in the ethidium bromide-stained gel (A). In contrast to *P. pijperi* and *P. jadinii*, the termini of *W. mrakii* mtDNAs appear to be mostly in the opened form. The closed form is detected here by the use of labeled probes. Sizes are indicated in bases.

ethidium bromide staining. As shown in the accompanying report (Fig. 4 in reference 8), these components corresponded to three derivative forms of terminal sequences; the first was a palindromic association of two right termini (head-head junction), the second was also a palindromic dimer of two left termini (tail-tail), and the third was a right-left (head-tail) junction. This interpretation was confirmed by the presence of the expected restriction sites and hybridization of right- and left-specific probes (sequences adjacent to the inverted terminal repeats) (Fig. 9). These three minor components were detected in all three species examined. To determine the nature of the junction point, the right-right junction fragment from *W. mrakii* was cloned and sequenced. As expected, a unique sequence of 379 bp was found between the inverted terminal repeat sequences (Fig. 5b). A single-stranded segment of 21 bases from the unique sequence was then chemically synthesized, labeled, and hybridized with *W. mrakii* mtDNA digests. The probe hybridized with the two extreme ends of the mtDNA, demonstrating that the cloned unique sequence existed as a part of the terminal structure. We may suppose that this 379-bp sequence forms the terminal hairpin of *W. mrakii* mtDNA as in the case of *P. pijperi*, but we failed so far to clone and sequence directly the termini of this DNA. The terminal loop sequence from *P. pijperi* hybridized to all three junction components of *P. pijperi* mtDNA digests. This finding suggested that the loop sequence is present at the apex of all three junction fragments of *P. pijperi* mtDNA, as in *W. mrakii* mtDNA. The terminal unique sequences are highly rich in adenine and thymine bases, 90% in *P. pijperi* 92% in *W. mrakii*. In the latter case, the sequence has a few repetitive sequences of 22 to 35 bases with 80 to 100% identity.

Possible sites of replication origins. To test whether the region of terminal loop sequences is capable of autonomous replication, the loop DNA and adjacent regions from *P. pijperi* and *W. mrakii* mtDNAs were inserted into the autonomously replicating sequence-less plasmid YIp5 and transformed into *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Both segments were indeed capable of supporting replication of the plasmid in these yeasts (data not shown; see Discussion).

DISCUSSION

A hairpin loop defines the ends of the linear mtDNA. With *P. pijperi* mtDNA, we have shown that a major fraction of the terminal fragments contained a closed hairpin loop. A variable fraction seems to be derived from this hairpin structure opened by a single-strand nick at a precise point that occurs within the inverted repeat sequence adjacent to the loop. A second form of the termini (shadow) seen in restriction digests likely corresponds to a nicked hairpin, although we do not know the exact configuration of this component in other DNAs.

With *W. mrakii* mtDNA, alkali denaturation of terminal fragments generated several fragments of defined lengths. Their size did not, however, correspond to that of the supposed hairpin loop. It is possible that single-stranded ends suffered degradation during DNA preparation in this particular case.

The terminal single-stranded loops have either of the complementary sequences with apparently equal probability. The flip-flop interconversion may be mediated by several mechanisms involved in replication, as discussed below.

Terminal inverted repeats. At the base of the terminal loops, there are long, precisely inverted repeats that form the symmetrical ends of the linear genome. These sequences do not seem to code for any protein and show, like the

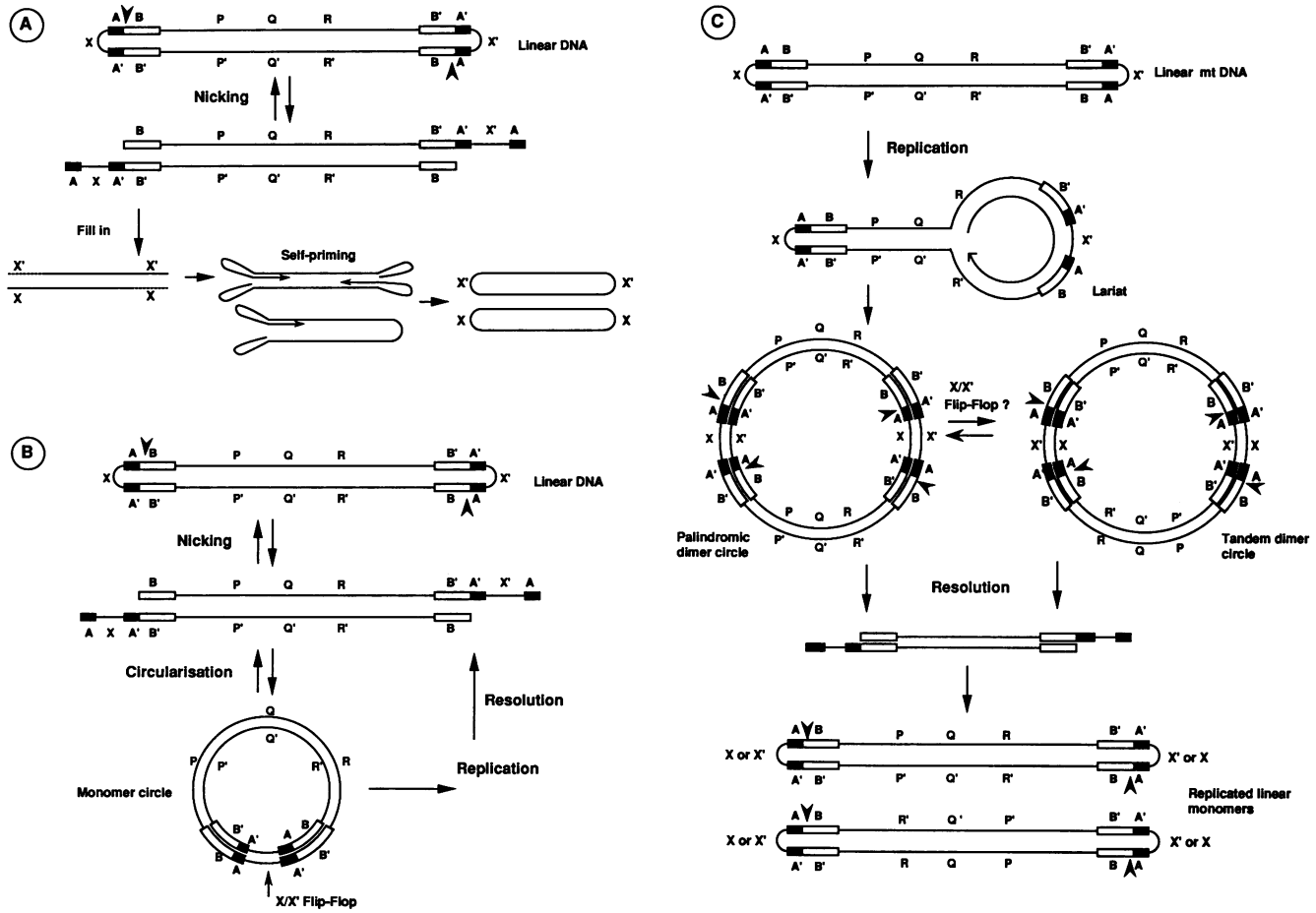

FIG. 10. Replication schemes. (A) Replication by self-priming; (B) replication through a monomer circle; (C) replication through a dimer circle. Boxes represent the inverted terminal sequence; arrowheads indicate single-strand incision points; A and A', B and B', etc., indicate pairs of complementary strands.

terminal loops, no similarity among *P. pijperi*, *W. mrakii*, and other linear mtDNAs examined. The terminal inverted repeats contain several internally repetitive motifs. In *W. mrakii*, there are long direct repeats (a 120-bp sequence repeated twice), several direct repeats longer than 20 bp, and a few palindromic repeats of 10- to 14-bp hemimer unit. In *P. pijperi*, one finds several short internal repetitions, the largest being a 15-bp unit repeated twice. The presence of these repeats is reminiscent of the flip-flop recombinase target region in the inverted repeats of the 2μm family plasmids (9).

Possible schemes for replication of linear mtDNA. The terminal structure of the linear mtDNA of yeasts appears to be different from those of the well-studied linear mtDNA of paramecia (11, 28, 29) and *Tetrahymena thermophila* (26). In paramecia, one end of the molecule has a loop and the replication starts near this loop to form a linear palindromic dimer molecule as an intermediate. In *T. thermophila*, the termini of unfixed length are made of many direct repeats. The properties of yeast linear mtDNA termini look rather like the termini described for vaccinia virus DNA. This large cytoplasmic genome also has flip-flopping AT-rich terminal loops next to large inverted repeats. Proximal to the loops, a nick appears to occur within the inverted repeats. DNA fragments representing palindromic junctions of termini have also been detected in this case.

Several types of replication mechanism can be envisaged for the linear mtDNA of yeasts. A complication in the case of yeasts is the fact that head-to-head, tail-to-tail, and head-to-tail junctions are all detected. Any model should take into consideration the presence of these junctions.

(i) With respect to vaccinia virus-type models of replication, Geshelin and Berns (10) have shown that vaccinia virus DNA has cross-linked termini. Baroudy et al. (1) have suggested several models for the replication of this linear DNA with hairpin ends. One model supposes self-priming after opening of the terminal hairpins, essentially following the Cavalier-Smith–Batesman scheme (2, 4) (Fig. 10A). When the replication goes through the closed hairpin of the opposite end, a double-stranded head-to-head or tail-to-tail junction is produced. After resolution, one recovers inverted hairpins. In vaccinia virus DNA, a specific short sequence necessary for resolution has been identified within the inverted repeats (24). Circular forms are not involved in this scheme. Head-to-tail junctions are not expected to appear but may be generated if there is concatemerization of the opened monomer ends.

(ii) Another model postulates replication through a monomer circle. Assuming that the left and right terminal loops are always complementary, their nick-opened forms can pair intramolecularly to form a monomer circle through a head-to-tail junction (Fig. 10B). Under the electron microscope,

P. pijperi mtDNA showed indeed the presence of a small number of double-stranded monomer-length circles. The circle may replicate through any mechanism used by circular mtDNA. This circular scheme accounts for the presence of a head-to-tail (right-left) junction and the presence of the monomer circles. We postulate that in this circular molecule, the junction segment undergoes flip-flop inversion between the inverted terminal repeat sequences. This flip-flop reaction may form part of the resolution process similar to the case of the 2μm plasmid replication as suggested by Futcher (9). This scheme, however, does not explain the origin of the head-to-head or tail-to-tail junction fragments. Such junctions may be generated by intermolecular recombination of monomer molecules at the inverted repeats, forming a palindromic dimer circle, as described for some chloroplast DNA (19).

(iii) In another model involving a circular intermediate, replication starts by de novo RNA priming within or near the terminal loop (Fig. 10C). This will form an entirely palindromic dimer circle containing both head-to-head and tail-to-tail junctions. Single-strand nicks near the base of the junction will resolve the replicated DNA into two monomeric linear molecules. We postulate again a flip-flop inversion reaction between the pairs of inverted terminal repeats within the dimer circle, so that both head-to-tail and head-to-head (tail-to-tail) junctions are generated (inversion of PQR/P'Q'R'). By electron microscopy, the dimer-length circles were found but very rarely, even less frequently than the monomer circles (4 dimer circles and 29 monomer circles were seen among 800 molecules).

Regardless of the type of model, resolution requires that a nick be introduced within the inverted repeats proximal to the double-stranded junction sequence. In *P. pijperi* and *P. jadinii* mtDNAs, the nicks indeed appear to occur within the inverted terminal repeat.

Concatemeric multimers, either direct or palindromic, are known to occur frequently in the [*rho*$^-$] mtDNAs of *S. cerevisiae* (33). So far we have no evidence for the existence of long concatemeric molecules. Rarely, very large linear molecules were observed by electron microscopy, but their nature is unknown.

Relationship with circular mtDNAs of yeasts. Although we cannot decide which model is most correct, the models involving circular intermediates offer a few preferable features. (i) They do not radically deviate from the replication of many circular genomes of yeasts, the linear form being considered a nonreplicative form derived from the circle. (ii) The flip-flop inversion thought to occur in the circular models is frequently seen in other circular genomes, including the 2μm family plasmids and many organelle DNAs; this inversion reaction may mediate the circular-linear conversion.

Our study showed that two yeast strains classified into closely related species on the basis of known criteria of taxonomy can differ in the form of mtDNA, one linear and the other circular. As we have shown in the accompanying report (8), *W. suaveolens* CBS 1670 mtDNA is linear, whereas *W. suaveolens* CBS 255 has a circular mtDNA. *W. beijerinkii* and *W. saturnus* strains have all linear mtDNAs, whereas *W. californica* and *W. pratensis* mtDNAs are circular. It is difficult to conceive that such related strains replicate their mtDNAs by completely different processes such as circular replication versus linear self-primed replication. More likely, the linear and circular forms of yeast mtDNA are replicated by essentially similar devices, and in some species or strains, the replicated molecules are converted into the linear form for unknown reasons. This conversion may not be an all-or-none process; the extent of conversion could vary according to the species. There may be a situation in which both linear and circular mtDNAs are present in comparable proportions, as seems to be the case in the fungus *Pythium* sp. (22). Note that when circular and linear forms are both present in an mtDNA preparation, the circular molecules tend to escape from detection under our pulsed-field gel electrophoresis conditions.

The presence of large inverted repeats in circular mtDNAs has also been reported in a number of microorganisms, including oomycetes (3, 16, 23, 31) and yeasts (5, 15, 37). Flip-flop inversion between the inverted repeats is known to occur in some of these genomes.

Possible replication origins. In *S. cerevisiae* mitochondria, transcription generally starts at the sequence ATATAAGTA or its close variants (34). In other yeast species (*K. lactis* [34] and *Candida glabrata* [6]) also, this motif seems to be commonly used for transcription of rRNA, tRNA, and mRNA as well as for transcription at the replication origins. In *P. pijperi* mtDNA, this motif was found in the 5′ flanking region of a tRNA gene cluster (EMBL accession number X66593) and in *W. mrakii* before the genes coding for cytochrome oxidase subunit II and apocytochrome *b*, two unassigned open reading frames, as well as a tRNA gene cluster (EMBL accession numbers X66594 and X66595). Interestingly, this motif occurs twice (in two orientations) within the terminal loop of *P. pijperi* and once in the loop of *W. mrakii*, suggesting that they can be the start sites of RNA-primed replication. The segment containing these motifs showed in fact that they were capable of supporting autonomous replication of a bacterial plasmid in yeasts. Unfortunately, these experiments do not indicate whether the loop sequences actually function as replication origins in mitochondria. It has been known that many AT-rich sequences from various sources exhibit an autonomously replicating activity in *S. cerevisiae* hosts (12, 18, 36).

Relationship with other organelle DNAs. Palmer and Thompson (27) have found that plant chloroplasts DNAs, usually circular, can be classified into two categories: one with a pair of large inverted repeats the other without. Intramolecular recombination appears to occur between the inverted repeats, leading to segment inversion (19). According to Palmer and Thompson (27), the sequence organization in the chloroplast DNA with inverted repeats is relatively constant compared with the other category of DNA, suggesting that the presence of the large inverted repeats may confer an evolutionary stability of gene organization on the organelle. The constant gene order found in the linear mtDNAs of yeasts might be correlated with this proposition. In the linear mtDNAs from the green algae *Chlamydomonas reinhardtii* (13) and the slime mold *Physarum polycephalum* (17, 35), it had also been noted that the sequence organization was highly constant among a number of independent isolates.

The case of *C. reinhardtii* is of particular interest (13, 14, 25, 30). Although the isolated mtDNA is essentially linear and contains inverted terminal repeats, early electron microscopic studies revealed also the presence of supercoiled circular molecules of similar size (13). These authors therefore speculated that the linear molecules are derived from circles by site-specific cleavage. Although the detailed structure of the termini of *Chlamydomonas* mtDNA is still not available, analysis of such cases may help to examine the general implication of the supposed linear-circular conversion process in organellar genomes.

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